

Properties of the interaction of fluoride- and guanylyl-5'-imidodiphosphate-regulatory proteins with adenylate cyclase

(trypsin-sensitivity/heat-inactivation/cyclic AMP/membrane-reconstitution/hormone regulation)

MATTHEW HEBDON*, HARRY LE VINE III*, NAJI SAHYOUN*, CLAUS J. SCHMITGES*, AND PEDRO CUATRECASAS

Department of Molecular Biology, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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ABSTRACT The mechanism of activation of adenylate cyclase by guanylyl-5'-imidodiphosphate [Gpp(NH)p] and NaF has been investigated by studying the reconstitution of Gpp(NH)p and NaF sensitivity of an enzyme rendered insensitive to these agents by differential detergent extraction of a particulate brain enzyme. Such reconstitution can be achieved by the addition of macromolecular regulatory factors from membranes of various tissues. Trypsin digestion and thermal inactivation provide evidence for the existence of two distinct regulatory functions, one capable of restoring the Gpp(NH)p response and another the NaF response. The regulatory protein(s) seem to interact with their respective activators in an easily reversible, divalent cation-independent reaction. This appears to be followed by a high-affinity interaction between the catalytic and regulatory components of adenylate cyclase in a slow, temperature-dependent, divalent cation-dependent process that produces the persistently activated state of the enzyme. The enzyme activation can be reversed by methods that separate catalytic from regulatory components and the resulting enzyme activity can be restimulated by the reconstitution technique.

The molecular mechanisms of activation of adenylate cyclase by NaF and guanylyl-5'-imidodiphosphate [Gpp(NH)p], and the relevance of such mechanisms to the hormonal modulation of this enzyme, have been difficult to investigate due to the lack of appropriate techniques for the resolution and reconstitution of the various molecular components of the adenylate cyclase system. We have reported (1) the development of such a technique which involves the preparation of a Gpp(NH)p- and NaF-insensitive brain particulate adenylate cyclase by differential detergent extraction. The enzyme regained its characteristic Gpp(NH)p and NaF responses upon the addition of one or more proteins solubilized from membrane preparations of various tissues (1). These regulatory proteins can be partially separated from solubilized adenylate cyclase by gel filtration on Ultrogel AcA 34 columns. We report here the utilization of this reconstitution technique to help elucidate the mode of interaction of the various components of this multifactorial system. Thermal and trypsin inactivation of the Gpp(NH)p- and NaF-reconstituting activities suggests that the two activities are functionally separable. Gpp(NH)p and NaF appear to interact reversibly with the corresponding regulatory proteins in a divalent cation-independent step, followed by a divalent cation-dependent step that leads to the persistent state of enzyme activation. Techniques that separate regulatory from catalytic components can also reverse the enzyme activation.

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MATERIALS AND METHODS

All tissues were from Sprague-Dawley rats (120 to 170 g). [α - 32 P]ATP, cyclic [2,8- 3 H]AMP and [8- 3 H]Gpp(NH)p were procured from ICN. Nonidet P40 was obtained from Shell; Lubrol PX was from ICI. Trypsin and soybean trypsin inhibitors were from Worthington and phospholipase C (*B. cereus*) was from Sigma.

The Gpp(NH)p- and NaF-insensitive adenylate cyclase was prepared by a modification of a published procedure (1, 2). Rat brain was homogenized with eight passes of a motor-driven glass/Teflon homogenizer in 8 vol (vol/wt) of 50 mM Tris-HCl, pH 7.6/0.25 M sucrose/3 mM dithiothreitol/3 mM MgCl₂; the 3000 \times g pellet was washed by repeating the same procedure, and the material was then suspended with a vortex mixer in 4 vol (vol/wt) of 50 mM Tris-HCl, pH 7.6/50% (vol/vol) glycerol/10 mM KCl/10 mM NaCl, diluted 1:8 in 50 mM Tris-HCl (pH 7.6), and centrifuged at 40,000 \times g for 10 min. This pellet was similarly suspended in 4 vol (vol/wt) of 50 mM Tris-HCl, pH 7.6/2 M sucrose, diluted 1:8 in 50 mM Tris-HCl (pH 7.6), and centrifuged at 40,000 \times g for 10 min. The pellet was then extracted with eight passes of a glass/Teflon motor-driven homogenizer in 8 vol (vol/wt) of 50 mM Tris-HCl, pH 7.6/0.5% Nonidet P40, 10% (vol/vol) glycerol/3 mM MgCl₂/3 mM dithiothreitol/0.4 mM ATP. The extract was incubated for 40 min at 37° and centrifuged at 40,000 \times g for 10 min. The pellet was similarly extracted in 1% Lubrol PX/3 mM dithiothreitol/50 mM Tris-HCl and the resulting pellet was extracted in 4 vol (vol/wt) of 3% Lubrol PX/50 mM Tris-HCl, pH 7.6, without incubation at 37°. The final pellet was suspended with eight passes of a glass/Teflon motor-driven homogenizer in 2 vol (vol/wt) of 50 mM Tris-HCl. Adenylate cyclase activity in this final preparation accounts for about 20% of the total original enzyme activity of the pellet, and it is remarkably stable for several hours at 30°.

A special technique for studying the preactivation of adenylate cyclase was developed which permits the use of high concentrations of fat cell particulate fractions solubilized in 50 mM Tris-HCl, pH 7.6/0.7% Lubrol PX (1) as a source of reconstituting activity without interfering with the enzyme assay itself. Adenylate cyclase and regulatory component preparations were incubated with various additions in a total volume of 100-150 μ l in 12 \times 75 mm glass test tubes, and 1 ml of ice-cold 50 mM Tris-HCl (pH 7.6) was added to stop the reaction and wash the enzyme membrane preparation; the latter was subsequently recovered quantitatively by centrifugation for 10 min at 5000 \times g in an RC-3 Sorvall centrifuge. The supernatant was aspirated and the pellet was suspended in 150 μ l of a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 0.2

Abbreviation: Gpp(NH)p, guanylyl-5'-imidodiphosphate.

* Listed according to alphabetical order.

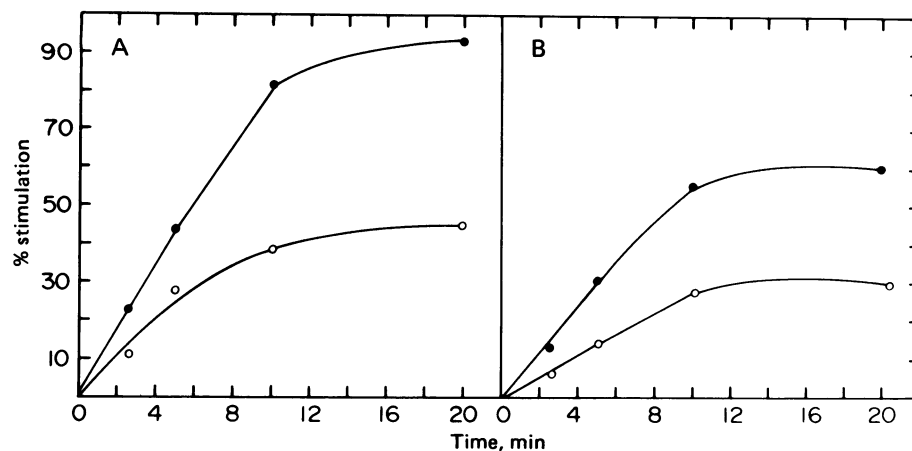


FIG. 1. Time course of the activation of reconstituted adenylate cyclase. The reconstituted system (2 ml) contained 5 mM MgCl_2 , 500 μg of protein of detergent-extracted brain membranes per ml, and 300 μg (●) or 150 μg (○) of protein of solubilized fat cell membranes per ml with 100 μM Gpp(NH)p (A) or 10 mM NaF (B). Aliquots (100 μl) were removed at various time intervals and treated according to the preactivation technique. Percentage stimulation was calculated relative to control activity of enzyme preincubated in the absence of Gpp(NH)p or NaF.

mM ATP, $2\text{--}4 \times 10^6$ cpm of [$\alpha\text{-}^{32}\text{P}$]ATP, 10 mM MgCl_2 , 7 mM phosphoenolpyruvate, 0.5 μl of pyruvate kinase, 10 mM aminophylline, and $7\text{--}9 \times 10^5$ cpm of cyclic [^3H]AMP. The reaction was stopped after 30 min at 30° by placing in a boiling water bath for 1 min, and the ^{32}P -labeled cyclic AMP formed was separated on neutral alumina columns (3). The reconstitution of the NaF and Gpp(NH)p responses of adenylate cyclase measured in this way usually represented a stimulation of about 2-fold. Basal activity was 80–120 pmol of cyclic AMP per mg of protein per min. All determinations were in duplicate or triplicate with a range of $\pm 7\%$.

The pure Ca^{2+} -dependent regulatory protein was kindly provided by Thomas C. Vanaman, Duke Medical Center. Actin and actomyosin prepared from platelets or chicken gizzards by high-salt extraction were obtained from Vann Bennett, Wellcome Research Labs.

RESULTS

In the reconstitution system described previously (1), four components were required for adenylate cyclase activation by Gpp(NH)p or NaF: the activator itself, a divalent cation such as Mg^{2+} , adenylate cyclase of the detergent-extracted rat brain particulate fraction, and one or more regulatory proteins present in solubilized fat cell particulate fractions. The activation of the enzyme in this composite system did not take place at 0° and occurred over 15 min at 30° (Fig. 1). The regulatory protein(s) did not lose more than 25% of their activity after 20 min at 30° under the assay conditions described in the legend to Fig. 1. The rate and the degree of stimulation depended on the concentration of regulatory protein(s) (Figs. 1 and 2) but seemed to be independent of the adenylate cyclase concentration above 200 μg of brain membrane protein per ml (Fig. 3). Below 200 $\mu\text{g}/\text{ml}$, the degree of activation increased steeply. Depletion of regulatory protein(s) did not exceed 20% even at high brain membrane protein concentrations and, therefore, does not account for the kinetics of the adenylate cyclase concentration curve.

This multifactorial reconstitution system was analyzed by varying the order of additions of its various components. Table 1 shows that all components must be present simultaneously to produce adenylate cyclase activation. However, this does not rule out *rapidly reversible* interactions between any two or more components that may not be detected by the experiments described in Table 1 because these could permit dissociation

reactions during the washing or separation procedures. Evidence for such rapidly reversible interactions involving the regulatory proteins and Gpp(NH)p or NaF was obtained by studying their inactivation by heat and trypsin.

Fig. 4A shows the time-dependent inactivation of the NaF-reconstituting activity at 30° and the stabilization of this activity in the presence of 10 mM NaF. Fig. 4B shows similar results for the Gpp(NH)p-reconstituting activity. Both activities decay with about the same half-time and can be similarly stabilized. However, Table 2 clearly demonstrates that NaF stabilizes the NaF-reconstituting activity but not that corresponding to Gpp(NH)p, whereas Gpp(NH)p stabilizes its own reconstituting activity but not that of NaF. Table 1 also shows that the regu-

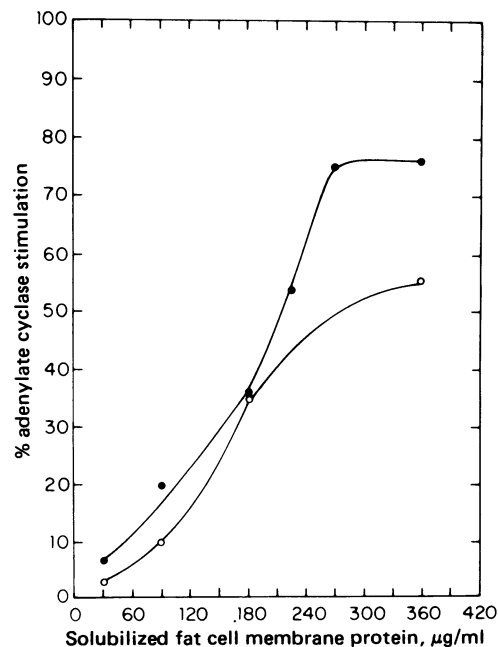


FIG. 2. Dependence of adenylate cyclase activation on the concentration of regulatory proteins. Various concentrations of solubilized fat cell membranes were incubated with 5 mM MgCl_2 and 500 μg of protein of detergent-extracted brain membranes per ml in the presence of 100 μM Gpp(NH)p (●) or 10 mM NaF (○) for 30 min at 30° , in a total volume of 130 μl . Samples were processed and percentage stimulation was calculated as described in the legend to Fig. 1.

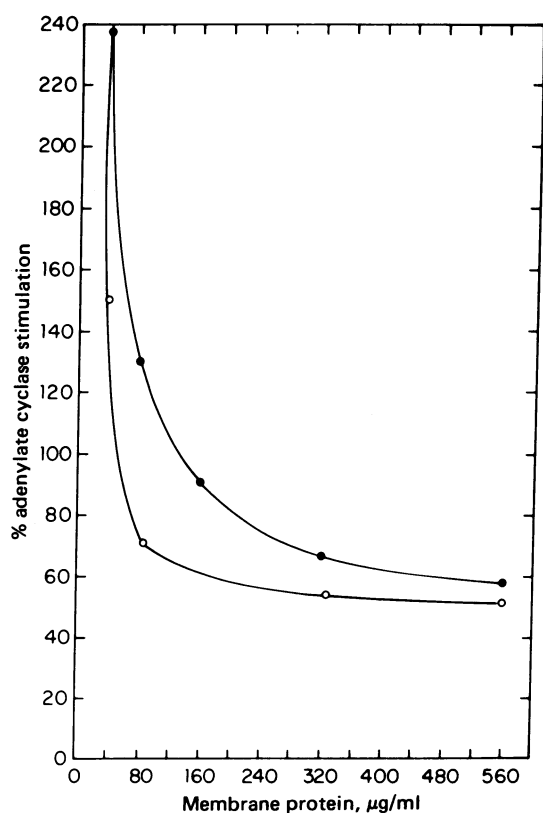


FIG. 3. Dependence of adenylate cyclase activation on the concentration of detergent-extracted brain membranes. Various concentrations of detergent-extracted brain membranes were incubated in the presence of 5 mM $MgCl_2$ and 300 μg of protein of solubilized fat cell membranes per ml in the presence of 100 μM Gpp(NH)p (●) or 10 mM NaF (○), in a total volume of 130 μl . Samples were subsequently treated as in Fig. 2.

latory components are not activated after removal of free Gpp(NH)p or NaF by gel filtration. $MgCl_2$ is not required for the apparent interaction between NaF or Gpp(NH)p and the respective regulatory components. Omission of divalent cations in the above experiments allows the study of the interactions between Gpp(NH)p or NaF and the regulatory protein(s) in the absence of adenylate cyclase activation in the solubilized fat cell membranes.

Fig. 5 shows that, in the *solubilized* form, both regulatory components are trypsin sensitive, that the NaF component is

Table 2. Lack of cross-stabilization against thermal inactivation of the NaF- and Gpp(NH)p-regulatory components

Additions during inactivation	% stimulation		
	No additions	Gpp(NH)p (100 μM)	NaF (10 mM)
None	0	55	62
Gpp(NH)p (10 μM)	5	90	60
NaF (10 mM)	0	50	92

Aliquots (0.3 ml) of solubilized fat cell particulate fraction were incubated for 30 min at 30° with 5 mM $MgCl_2$, 0.2 mg of phenylmethylsulfonyl fluoride per ml, and 3 mM dithiothreitol in the absence or presence of 10 mM NaF or 10 μM Gpp(NH)p. The incubation was terminated by applying the aliquots to 3-ml Sephadex G-50 (medium) columns followed by elution with 50 mM Tris-HCl, pH 7.6/ $MgCl_2$ phenylmethylsulfonyl fluoride/dithiothreitol at the same concentrations as above. The void volumes were collected and assayed for Gpp(NH)p- and NaF-reconstituting activities, which are expressed as percentage of control activities in solubilized fat cell particulate fraction kept at 4° and then passed on a Sephadex column. By using [8- 3H]Gpp(NH)p, it was estimated that <1% of the Gpp(NH)p applied to the columns appeared in the breakthrough volume.

more sensitive than the Gpp(NH)p component, and that the corresponding regulatory components become significantly more sensitive to trypsin digestion in the presence of Gpp(NH)p or NaF. Neither regulatory component was affected by treating fat cell membranes with trypsin (100 μg /ml, 10 min at 30°) or phospholipase C (2 units/ml, 10 min at 30°) whereas adenylate cyclase activity was decreased by 70–80%, providing further evidence that the regulatory component(s) is distinct from the catalytic moiety and that the former is protected in the cell membrane against tryptic digestion.

We attempted to provide direct evidence for “binding” of the regulatory components to the adenylate cyclase preparation in the presence of $MgCl_2$ and NaF or Gpp(NH)p. Only in the presence of relatively high concentrations of the adenylate cyclase preparation (1.5 mg of protein per ml) was it possible to detect a 15–20% NaF- or Gpp(NH)p-dependent loss of reconstituting activity, indicating that, even when the enzyme is fully activated, most of the regulatory protein(s) remain “free” in solution. However, the results of another experimental approach (Fig. 6) provide indirect evidence for a “binding” event: the activated state of the enzyme can be reversed by extraction with detergent at 37° and restimulation requires the addition of regulatory protein(s). The addition of Gpp(NH)p or NaF alone does not produce restimulation.

Isoproterenol (50 μM), glucagon (10 μM), and insulin (0.1–1 milliunit/ml) had no effect on the activity of the regulatory

Table 1. Effects of varying the order of additions on the enzyme activation in the reconstituted system

Additions during first incubation	Additions during second incubation	% adenylate cyclase stimulation
Enzyme + regulatory protein(s) + $MgCl_2$ + Gpp(NH)p	50 mM Tris-HCl, pH 7.7	120
Enzyme + regulatory protein(s) + $MgCl_2$	Gpp(NH)p + $MgCl_2$	0
Enzyme + Gpp(NH)p + $MgCl_2$	Regulatory protein(s) + $MgCl_2$	7
Regulatory proteins + Gpp(NH)p + $MgCl_2$	Enzyme + $MgCl_2$	4
Enzyme + Gpp(NH)p + $MgCl_2$ (A)	A + B	8
Regulatory proteins + Gpp(NH)p + $MgCl_2$ (B)		
Fat cell membranes + Gpp(NH)p + $MgCl_2$ followed by solubilizing the membrane preparation	Enzyme + $MgCl_2$	0

“Enzyme” refers to detergent-extracted brain membranes. “Regulatory proteins” refers to solubilized fat cell membranes. The concentrations of $MgCl_2$ and Gpp(NH)p were 5 mM and 100 μM , respectively. The first incubation was carried out in a volume of 100 μl for 15 min at 30°. When “Enzyme” or fat cell membranes were used, the reaction was stopped by washing in 1 ml of 50 mM Tris-HCl (pH 7.7). When “Regulatory proteins” were used, the reaction was terminated by gel filtration on a 3-ml Sephadex G-50 (medium) column. The second incubation lasted 30 min at 30°, in a volume of 100 μl , and the samples were subsequently treated according to the preactivation technique. Similar results were obtained when 10 mM NaF was substituted for Gpp(NH)p.

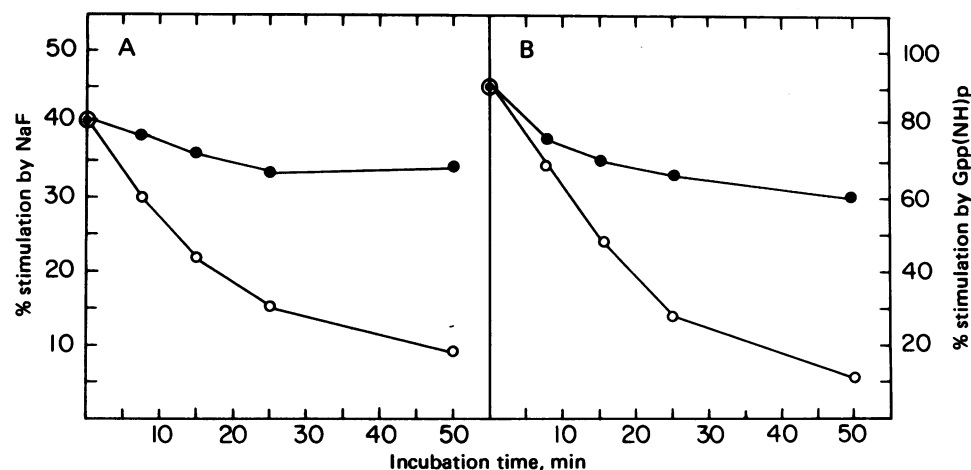


FIG. 4. Stabilization of the NaF- and Gpp(NH)p-regulatory components against thermal inactivation (A and B, respectively). Aliquots (0.4 ml) of solubilized fat cell particulate fraction (400 μ g of protein per ml) were incubated for various periods of time at 30° in the absence (O) or presence (●) of 10 mM NaF or 100 μ M Gpp(NH)p. All incubations contained 0.2 mg of phenylmethylsulfonyl fluoride per ml and 3 mM dithiothreitol; 50- μ l portions from each sample were used for adenylate cyclase preactivation using 100 μ M Gpp(NH)p and 10 mM NaF, respectively, and 300 μ g of protein of extracted brain membranes per ml.

components, whether the hormones are added directly to the reconstituted system or to the fat cell membrane preparation prior to solubilization. Moreover, the calcium-dependent regulatory protein (100 μ g/ml) and platelet or smooth muscle actin or actomyosin did not influence the reconstitution. Cytochalasin B (1 μ M) or colchicine (0.1 mM) also had no effect.

DISCUSSION

We recently reported the occurrence of a membrane-bound regulatory protein(s) that mediates the stimulation of adenylate cyclase by Gpp(NH)p and NaF (1). This protein(s) is not solubilized with 0.1 mM EDTA, 0.5–1 M KCl, or 4 M urea (un-

published data) but, after detergent solubilization, it can reconstitute the Gpp(NH)p and NaF sensitivity of adenylate cyclase preparations that have been previously rendered non-responsive to these activators (1). The action of this regulatory protein(s) does not exhibit tissue or species specificity. Others have reported different approaches for the separation of the adenylate cyclase system into regulatory and catalytic components with subsequent reconstitution of the Gpp(NH)p and NaF responses. Such approaches include the use of affinity chromatography on guanylyl nucleotide-Sepharose columns (4) and the use of cell lines defective in one or more components of the adenylate cyclase system (5, 6). However, the nature of the molecular interactions between these various components

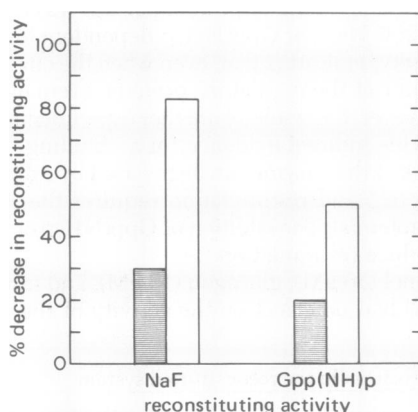


FIG. 5. Trypsin-sensitivity of the NaF- and Gpp(NH)p-regulatory components. Aliquots (0.4 ml) of a regulatory component preparation (400 μ g of protein/ml) were incubated for 10 min at 30° in 3 mM dithiothreitol/5 mM $MgCl_2$ /10 μ g of trypsin per ml in the presence (open bars) or absence (hatched bars) of 100 μ M Gpp(NH)p or 10 mM NaF. The protease digestion was arrested by the addition of an equal amount of soybean trypsin inhibitor. Controls were incubated for a similar period of time at 30° except that trypsin and soybean trypsin inhibitor were mixed together prior to addition to the solubilized regulatory components. The reconstituting activity for controls and trypsin-treated preparations was assayed in the presence or absence of 100 μ M Gpp(NH)p or 10 mM NaF and 350 μ g of protein of extracted brain membranes per ml. The percentage decrease in reconstituting activity represents the fractional decrease of adenylate cyclase stimulation by the trypsin-treated regulatory components compared to "controls."

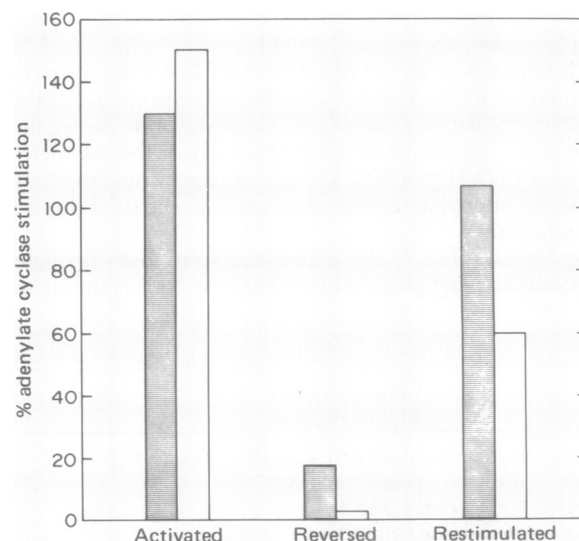


FIG. 6. Reversal and restoration of Gpp(NH)p and NaF activation of adenylate cyclase. Intact brain membrane preparations were activated by treatment with 100 μ M Gpp(NH)p (hatched bars) or 10 mM NaF (open bars). The activation was reversed by detergent extraction. The enzyme activity was restimulated by the addition of 100 μ M Gpp(NH)p (hatched bars) or 10 mM NaF (open bars) in the presence of 5 mM $MgCl_2$ and 300 μ g of protein of solubilized fat cell membranes per ml. Similar results were obtained when detergent-extracted brain membranes were initially substituted for intact brain membranes.

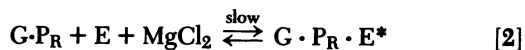
remains obscure. The relatively simple reconstitution system developed earlier (1) and further explored here helps to partially clarify the mode of these molecular interactions.

The differential trypsin sensitivity of the NaF- and Gpp(NH)p-reconstituting activities and the lack of cross-stabilization against thermal inactivation suggest that the reconstituting activities reside in separate regulatory proteins. Moreover, gel filtration chromatography of solubilized fat cell membranes (unpublished data) and trypsin or phospholipase C treatment of intact membranes indicate that these regulatory proteins are distinct from the catalytic component.

The protection against thermal inactivation indicates that Gpp(NH)p and NaF can interact specifically with their regulatory proteins in the absence of divalent cations. The increased sensitivity of the regulatory components to trypsin in the presence of Gpp(NH)p and NaF also supports the proposed specific interaction between the regulatory components and their corresponding activators. Furthermore, this interaction must be quickly reversible insofar as the regulatory components are not persistently activated after the removal of free Gpp(NH)p or NaF. In contrast, the properties of the reconstituted adenylate cyclase system indicate that the activation achieved is relatively persistent, is time-dependent over at least 15 min at 30°, requires MgCl₂, and does not occur at 0°.

We have also shown that the resultant activation can be reversed and subsequently restored by procedures that, respectively, separate and reconstitute the catalytic and regulatory components of the adenylate cyclase system, implying that the catalytic component is not covalently modified during activation. The "separation" of catalytic from regulatory components may be partly due to selective thermal inactivation of the latter. Furthermore, reversal of the Gpp(NH)p stimulation by the addition of relatively high concentrations of GTP (unpublished data) or GTP and isoproterenol (7, 8) suggests that Gpp(NH)p is bound noncovalently to the activated enzyme system. The kinetics of the time course of adenylate cyclase activation (Fig. 1) also support a nonenzymatic mechanism for the stimulation of adenylate cyclase activity by Gpp(NH)p or NaF.

The observations discussed thus far are consistent with the following model for the activation of adenylate cyclase:



in which P_R represents the regulatory component(s), E is the catalytic moiety (E^* is an activated state), and G is either Gpp(NH)p or NaF. On the basis of the fact that hormones in-

crease the rate at which Gpp(NH)p activates the enzyme (9, 10), it is suggested that hormones act at the second step when G is GTP or a GTP analogue. This second step may involve a fast binding process followed by a slow change in the catalytic subunit leading to the activated state of the enzyme. Because the activation of fat cell membrane adenylate cyclase by Gpp(NH)p or NaF does not decrease the yield of regulatory activity solubilized from these membrane preparations (unpublished data), it is suggested that the regulatory protein(s) is present in excess over the enzyme concentration. Furthermore, because activation of the brain enzyme reconstituted system leaves most of the regulatory protein(s) "free" in solution, step 2 may represent a high-affinity, low-capacity interaction. The above suggestions are based on a relatively crude system and purification or specific labeling of one or more constituents will further test these suggestions.

Other problems remain to be investigated, including the actual mechanism by which hormones exert their effect and any peculiar molecular properties of the regulatory proteins contributing to hormonal activation of adenylate cyclase. Although these regulatory proteins seem to be distinct from the Ca²⁺-dependent regulatory protein (11), their relationship to the putative hormone-sensitive GTPase (12) and to the Gpp(NH)p-sensitive retinal cyclic GMP phosphodiesterase (13) has not been elucidated.

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